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FGF signaling antagonizes cytokine-mediated repression of Sox9 in SW1353 chondrosarcoma cells

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Summary

Objective: The Sox9 transcription factor has emerged as an important determinant of chondrocyte differentiation, including the regulation of type II collagen (Col2) and aggrecan gene expression. We sought to identify a human cell line model that conserves the Sox9 regulatory pathways identified in the mouse.

Design: The SW1353 chondrosarcoma cell line was considered to be a candidate for Sox9 studies. The activity of a Sox9 regulated *Col2a1* enhancer reporter gene was analyzed in response to treating cells with known regulators of murine Sox9 expression/activity. The effect of treatment on expression of the endogenous Sox9 gene was analyzed by real-time PCR and Western blot.

Results: Col2 enhancer activity was stimulated by fibroblast growth factors (FGF-1 and -2) and repressed by inflammatory cytokines (IL-1β and TNFα) in SW1353 cells. These effects correlated with changes in Sox9 mRNA and protein levels. In addition, FGF-9 was shown to stimulate enhancer activity and Sox9 expression. Cotreatment studies demonstrated that FGFs functionally antagonize the cytokine-mediated repression of Sox9 expression and Col2 enhancer activity.

Conclusions: SW1353 cells represent a useful human cell model as they conserve many Sox9 signaling pathways previously demonstrated in mouse chondrocytes. We identify FGF-9 as a particularly potent Sox9 agonist. The antagonism between FGFs and cytokines on Sox9 expression and Col2 enhancer activity suggests that Sox9 integrates the opposing activities of FGFs and cytokines. We also find that SW1353 cells respond to very low doses of IL-1 with Col2 enhancer activation, while increasing doses lead to repression.

Key words: Sox9, Fibroblast growth factor, IL-1, TNF.

Introduction

Common to osteoarthritis (OA) and rheumatoid arthritis (RA) is the progressive destruction of articular cartilage. This tissue provides the lubrication and compressibility needed for joint function. Therefore, the loss of articular cartilage results in increasing morbidity and pain. Articular cartilage is composed of chondrocytes embedded in a collagen- and proteoglycan-rich extracellular matrix. Thus, the chondrocyte is central to understanding normal cartilage function as well as the arthritic and degenerative diseases that affect it. Isolated primary chondrocytes have little proliferative capacity and tend to de-differentiate to fibroblast-like cells when cultured *in vitro*. Sources of human cells for study, particularly from non-diseased tissue, are extremely scarce for obvious reasons. Therefore, much of our knowledge of chondrocyte biology is derived from animal studies or their cultured tissues and cells.

The genetic studies from human subjects suffering from severe skeletal abnormalities have contributed to our understanding of chondrocyte biology. Mutations leading to the loss of function or expression of a single allele of the human Sox9 gene were identified as the cause of campomelic dysplasia[1], a rare and severe skeletal malformation syndrome[2]. Male patients also present with XY sex reversal[1] confirming the other major role of Sox9 in development, male sex determination. Mice engineered to express only one allele of the Sox9 gene (Sox9 +/−), represent a near phenocopy of the human skeletal syndrome[3], displaying hypoplasia of all cartilage primordia and bones derived therefrom. Furthermore, mice derived by chimerism of wild-type and Sox9−/− embryonic stem (ES) cells showed that Sox9 is obligatory for cells to be incorporated into mesenchymal condensations destined to give rise to cartilage structures[4]. The role of Sox9 in cartilage determination is conserved across vertebrates, as ectopic expression of Sox9 in developing chick embryos showed the potential to trans-differentiate muscle and dermis into cartilage[5]. Genetically modified mice having impaired or enhanced Sox9 expression showed XY or XX sex reversal, respectively[6,7], and demonstrated the conservation of Sox9 in sex determination in mice and humans.

Sox9 is a member of a family of HMG-box proteins. This family of proteins is unusual in that they bind to the minor groove of DNA and cooperate with a protein partner(s) to regulate gene expression (reviewed in reference 8). LSox5 and Sox6 appear to play overlapping roles in collaborating with Sox9 to regulate the expression of chondrocyte target genes including *Col2a1* and aggrecan[9,10]. Sox9 also regulates the 'minor cartilage collagen', Col11a2, by interactions with HMG-like sites in both the promoter[11] and within an intronic enhancer[12]. Sox9 appears to integrate the many

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signaling pathways regulating the expression of cartilage matrix proteins. These include signals that positively regulate the cartilage phenotype, such as FGFs, and BMP-2, as well as parathyroid hormone related protein (PTHrP) in prehypertrophic chondrocytes[5,13,14]. Inhibition of matrix expression by cytokines and retinoic acid also involves inhibition of Sox9[15,16]. Such studies have shown that Sox9 activity is regulated by both the level of Sox9 expression and by post-translational modification[17]. Sox9 has been termed the 'Master Chondrogenic Transcription Factor'[15]. Therefore, understanding Sox9 function in cartilage development, tissue maintenance, and disease processes may be important in considering strategies to halt cartilage destruction, to maintain the chondrocyte phenotype, and to induce repair. Here, we show that the human SW1353 cell line conserves many of the Sox9 signaling pathways found in primary murine chondrocytes. We make the novel observation that FGF-9 stimulates Sox9 expression and Col2 enhancer activity. In addition, we show that very low levels of IL-1β actually stimulate expression of the Col2 enhancer, while higher levels are inhibitory. This finding may have relevance to the course of OA.

Materials and methods

SOX9 EXPRESSION PLASMID

To generate the human Sox9 mammalian expression plasmid, total RNA from cultures of immortalized human chondrocyte-like cells T/C-28a4[18] was purified using an RNeasy kit with DNase treatment (Qiagen). The entire 1.5 kb coding region (GenBank database, accession no. Z46629) was obtained by reverse transcription (Clontech Advantage RT Kit) followed by PCR reaction (Roche Expand High Fidelity PCR Kit) using primers: sense 5′-CGGGATCCGCCACCATGAATCTCCTGGACCCCTTCAT G-3′ and antisense 5′-CGGAATTCCTCAAGGTCGAGTG AGCTGT-3′. The PCR product was subcloned into pcDNA3.1 (+) (Invitrogen) and sequence verified.

CELL CULTURE

Tissue culture reagents were obtained from Invitrogen. Recombinant FGF-1, -2, -7, and -9, IL-1β and TNFα were obtained from R&D Systems. FGF-7 was supplied as a glycerin solution containing bovine serum albumin (BSA); all other growth factors and cytokines were supplied as lyophilized samples containing BSA and reconstituted in phosphate buffered saline (PBS) containing BSA according to the manufacturer's directions. SW1353 human chondrosarcoma cells (ATCC #HTB-94) were grown in DMEM supplemented with 10% fetal bovine serum and 10 µg/ml gentamicin. Subconfluent cells were passaged using 0.25% trypsin–EDTA, approximately 2 times per week by dividing 1:10.

TRANSFECTION EXPERIMENTS

Experiments involving cotransfection of the Sox9 expression plasmid with the 48 bp *Col2a1* luciferase reporter[19] were performed with 2.6×10^4 SW1353 cells plated in 24-well dishes. The next day 0.25 µg of 48 bp *Col2a1* reporter plasmid and a total of 0.25 µg of the Sox9 expression plasmid and/or pcDNA3.1 vector (Invitrogen) were added to 1 µl of TransIt (PanVera) diluted into 100 µl of OptiMEM. After 15–30 min, transfection complexes were diluted to 1 ml with OptiMEM. Growth media were removed and replaced with transfection mixture. Transfection complexes were removed after 7 h and replaced with fresh OptiMEM. Cells were harvested 48 h post-transfection and luciferase activity measured using LucLite (Packard) and a TopCount plate reader (Packard).

To assess the effects of growth factors and cytokines, differences in well-to-well transfection efficiency were minimized by performing bulk transfection of the 48 bp *Col2a1*[19] luciferase reporter. Twenty-four hours prior to transfection, 10^6 cells were plated in 10 ml of growth media in 75 cm^2 culture flasks. Each transfection consisted of 24 µl of TransIt (PanVera) diluted into 250 µl OptiMEM, followed by the addition of 12 µg of plasmid DNA. After 15–30 min of incubation, growth media were removed from the cells and replaced with the DNA–transfection reagent complexes diluted to a final volume of 10 ml with OptiMEM. After 7 h, each 75 cm^2 flask of cells was subcultured into a 96-well plate using Cell Dissociation Solution (Sigma) and OptiMEM. Twenty-four hours post-transfection, cells were treated with FGFs or cytokines in OptiMEM. Vehicle treated cells received an amount of PBS/BSA reconstitution buffer that was equivalent to the highest amount included with any FGF or cytokine treatment group (210 ng/ml BSA). Luciferase activity was measured 24 h after treatment with LucLite (Packard) or Bright-Glow (Promega) reagents.

HOECHST FLUOROCHROME ASSAY

Cells were plated into duplicate 96-well plates following transfection and treated as described earlier. Media were removed 24 h after treatment and cells were frozen in 100 µl of distilled water and lysed by a total of three freeze–thaw cycles. DNA was stained with 100 µl of 20 µg/ml Hoechst 33528 dye in TNE (100 mM Tris, pH 7.0, 10 mM EDTA, 2 M NaCl), overnight at 37°C. Staining was determined by reading fluorescence in a CytoFluor 4000 (Perspective Biosystems) with 360 nm excitation, 460 nm emittance. Cell number was determined using a standard curve.

WESTERN BLOTTING

Cell lysates were prepared from monolayer cultures in 24-well dishes by the addition of SDS sample buffer (Invitrogen). Equivalent amounts of lysate were sonicated to shear genomic DNA, heat denatured in the presence of 2-mercaptoethanol, and electrophoresed through SDS-polyacrylamide (4–20 or 12%) gels in Tris–glycine buffer (Invitrogen). Proteins were transferred to nitrocellulose (Invitrogen) using a semi-dry apparatus (BioRad) and the membrane blocked with Western blocking reagent (Roche). Sox9 antibody[20] was used at a 1:1000 dilution and GAPDH antibody (Advanced Immunochemical) at 1:5000 dilution. The secondary antibodies were goat anti-rabbit IgG and rabbit anti-mouse IgG, conjugated to HRP (Pierce). Super Signal Pico and Super Signal Femto ECL reagents (Pierce) were used to develop blots prepared with overexpressed or endogenous protein, respectively. Chemiluminescence was imaged with a Lumi-Imager (Roche). Sox9 and GAPDH band intensities were quantitated using the Lumi-Analyst software 1D-gel application (Roche). Gel loading differences were normalized to GAPDH values. Relative Sox9 was calculated by dividing the normalized Sox9 values by the normalized untreated control lane value and reported as fold change from the untreated control.

TAQMAN RNA ANALYSIS

RNA was prepared from cells grown in 75 cm^2 dishes using RNeasy Kits with DNase treatment (Qiagen) according to the manufacturer's protocol, except that a second application of DNase was included. cDNA was prepared from 10 to 20 µl of RNA (1–4 µg) using random hexamer primers and Taqman Reverse Transcription reagents (Applied Biosystems). Taqman reactions were performed with 8 µl cDNA and Universal PCR Master Mix (Applied Biosystems), 900 nM PCR primers (forward primer 5′-CACACAGCTCACTCGACCTTG-3′ and reverse primer 5′-TTCGGTTATTTTTAGGATCATCTCG-3′), and 200 nM probe (6-carboxyfluorescein [FAM]-5′-CCCACGAAGGGC GACGATGG-3′[TAMARA] 6-carboxytetramethylrhodamine). Reactions (50 µl) were run using a Perkin–Elmer 5700 under standard conditions for 45 cycles. The data were analyzed using GeneAmp5700 software (Applied Biosystems), with threshold adjusted to a linear range for all samples. The change in threshold cycle number (ΔCt) was normalized to the GAPDH reference gene (commercial probe and primers from Applied Biosystems), by subtracting ΔCt_{GAPDH} from ΔCt_{Sox9}. The effect of treatment (ΔΔCt) was calculated by subtracting $\Delta Ct_{control}$ from $\Delta Ct_{treated}$. Fold induction was determined by calculating $2^{\Delta\Delta Ct}$. Data are reported as percent target cDNA in a treated sample relative to untreated.

Results

COL2 ENHANCER ACTIVITY IS RESPONSIVE TO INCREASING LEVELS OF SOX9

We began our studies by analyzing the activity of the well-characterized 48 bp *Col2a1* enhancer construct (48 bp *Col2a1*[19]) in transfected SW1353 cells. This reporter plasmid is constructed of five tandem repeats of the 48 bp *Col2a1* enhancer and encompasses the minimal sequence, including Sox9 binding sites, sufficient to direct chondrocyte-specific expression in transgenic mice[21]. While other transcription factors bind to this enhancer fragment and help direct chondrocyte-specific gene expression (e.g., LSox5 and Sox6[10]), Sox9 binding is necessary for full activity[21].

SW1353 cells plated in 24-well dishes were transfected with a constant amount of reporter (0.25 µg), with or without increasing amounts of the Sox9 expression construct or empty vector. Basal luciferase expression was detected with the 48 bp *Col2a1* reporter alone [Fig. 1(A), see inset]. The enhancer-less p89 control plasmid, containing the same *Col2a1* minimal promoter, was essentially inactive (<5% activity, not shown). This suggests that SW1353 cells constitutively express transcription factors that drive *Col2a1* enhancer activity. However, we determined that Sox9 levels were limiting, as *Col2a1* enhancer activity was increased in a dose-dependent manner by cotransfection with increasing amounts of the Sox9 expression plasmid (Fig. 1A), and consistent with the results of cotransfection experiments performed in murine chondrocytes and BALB/3T3 cells[20]. At the highest concentration (0.25 µg), a slight reduction of reporter activity was observed. This 'squelching' is often seen when transcription factors are expressed at very high levels. Sox9 expression was confirmed by preparing Western blots from cell extracts prepared from duplicate transfections [Fig. 1(B)]. An immunoreactive band migrating with the 66 kDa marker was observed with increasing intensity in cells transfected with escalating amounts of Sox9 expression plasmid [Fig. 1(B)]. Expression of endogenous Sox9 was not detected in untransfected SW1353 cells under these conditions, but

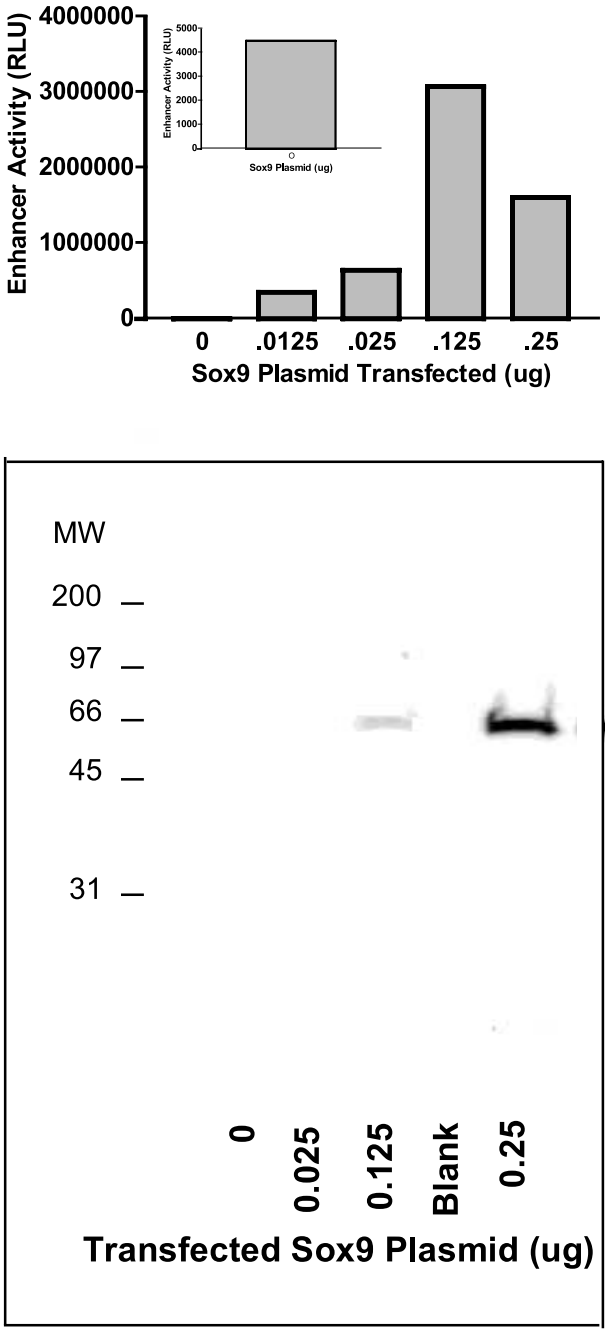

Fig. 1. Basal expression of the 48 bp *Col2a1*Luc enhancer reporter is stimulated by coexpression of Sox9 in transiently transfected SW1353 cells. Duplicate cultures of SW1353 cells were transfected with a constant amount of the reporter plasmid DNA (0.25 µg) and increasing amounts of Sox9 expression plasmid (0–0.25 µg) balanced with pcDNA3.1 vector plasmid (0.25–0 µg). Cell extracts were prepared from one of the duplicate cultures 24 h post-transfection and luciferase activity measured (A). The Y-axis is expanded in the inset to illustrate basal expression of the 48 bp *Col2a1*Luc reporter. Results from a single experiment are shown and are representative of those obtained in three independent studies. (B) Whole cell extracts were made from the corresponding duplicate cultures depicted in (A) and analyzed by Western blot using an antibody raised against Sox9.

endogenous Sox9 expression was observed using conditions of enhanced sensitivity to develop the blot (see Fig. 6 subsequently). These results suggest that endogenous expression of Sox9 is limiting, although sufficient to direct basal expression of the *Col2a1* enhancer in SW1353 cells.

SW1353 CELLS ARE RESPONSIVE TO PRO-CHONDROGENIC FGFS

FGF receptor signaling has a profound effect on the growth and differentiation of chondrocytes *in vitro*. However, the determination of physiologically relevant interactions is made enormously complex since there are four FGF receptors and 22 ligands in this family[22]. In murine chondrocytes and immortal chondrocyte cell-lines (ATDC5 and C3H10T1/2), the 48 bp *Col2a1* luciferase reporter is induced by pro-chondrogenic factors including FGF-1 and FGF-2, but not by FGF-7[14], which signals through the epithelial-cell restricted FGFR2IIIb receptor. We analyzed the responsiveness of SW1353 cells to these factors using the 48 bp *Col2a1* enhancer transfection assay. Addition of FGF-1 or FGF-2 to the cultures increased the 48 bp enhancer reporter activity approximately 3-fold, whereas FGF-7 expression had no effect [Fig. 2(A)]. Our results show the conservation of FGF-1 and FGF-2 signaling pathways in SW1353 cells.

We extended our studies to examine the effect of FGF-9 on enhancer activity. FGF-9 is a potent chondrogenic factor and signals through FGFR3b and FGFR2c[23]. Mutations that result in a constitutively active FGFR3 disturb the balance between chondrocyte growth and differentiation[24], leading to skeletal malformations including achondroplasia, hypochondroplasia, and thanatorphoric dysplasia[25–27]. Furthermore, FGF-9 overexpression has been implicated in chondromatosis, a rare disease characterized by cartilaginous nodule formation of the synovium[28]. These studies suggest a central role of FGF-9 in controlling the differentiation of stem cells into chondrocytes. When added to cultures of SW1353 cells that had been transfected with the *Col2a1* reporter, FGF-9 stimulated enhancer expression up to 4-fold [Figs. 2(A) and 3]. This result suggests that SW1353 cells express functional FGFR3b and/or FGFR2c receptors.

FGF family members are mitogenic in some cell types[22] and we considered the possibility that the effect of growth factor on luciferase activity might be attributed to differences in the numbers of cells recovered at the time the cultures were analyzed for luciferase activity. Therefore, we determined the numbers of SW1353 cells following each treatment in duplicate plates of transfected SW1353 cells using a sensitive Hoechst dye DNA staining procedure. As shown in Fig. 2B, after 24 h, similar numbers of cells were recovered from the FGF treated cultures and vehicle controls and argues against an indirect effect of FGFs on cell number as that being responsible for increased *Col2a1* activity.

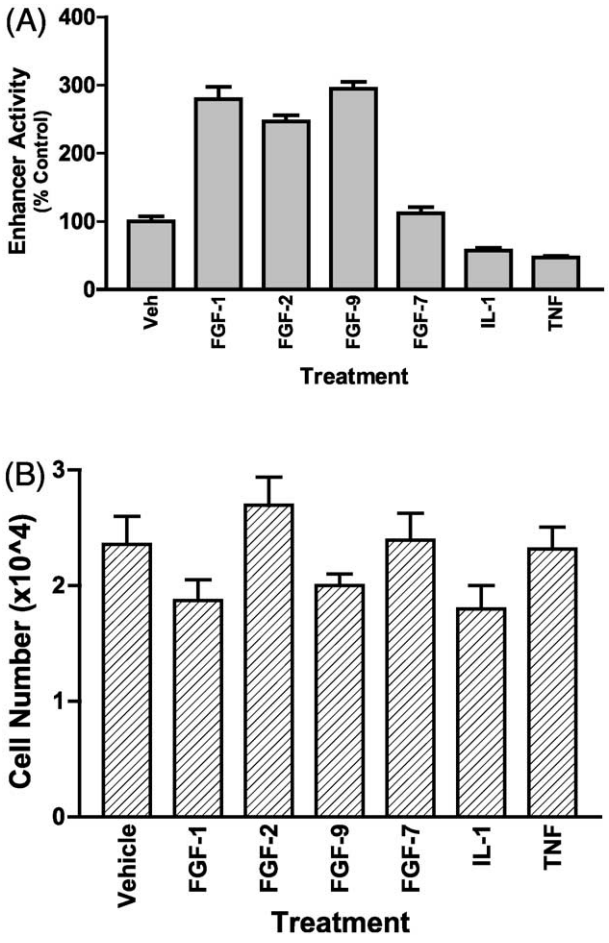

Fig. 2. SW1353 cells respond to FGFs or cytokines with activation or repression of the 48 bp *Col2a1* enhancer reporter without affecting cell numbers. To ensure equal transfection efficiency among all samples, 75 cm^2 flasks of SW1353 cells were transiently transfected in bulk with the 48 bp *Col2a1* enhancer luciferase reporter. Seven hours post-transfection cells were dissociated, pooled, and plated into duplicate 96-well plates. The next day cells were treated with FGF-1 (200 ng/ml), FGF-2 (5.9 ng/ml), FGF-9 (200 ng/ml), FGF-7 (200 ng/ml), TNFα (10 ng/ml), or IL-1β (10 ng/ml); these doses produced the maximal effect in dose–response assays (data not shown). Luciferase activity was determined 24 h post-treatment from one set of duplicate plates. Results represent the mean and standard error from a representative study; in this example 12 replicate wells for each treatment were used (A). The relative light units have been normalized to percent vehicle control. Similar results were obtained in three independent experiments. (B) Cell counts recovered from the duplicate plates of cultured cells in (A) were determined by DNA staining using Hoechst dye and a standard curve prepared with known numbers of SW1353 cells. The graph indicates the mean cell number and standard error for each treatment group that comprises 12 replicates.

PRO-INFLAMMATORY CYTOKINES REPRESS COL2 ENHANCER ACTIVITY IN SW1353

IL-1β and TNFα were shown to repress *Col2a1* enhancer activity in primary murine chondrocytes. This activity is NFκB-dependent and coincides with reduced Sox9 expression[15]. SW1353 cells express functional TNF and IL-1 receptors as these agents can stimulate the expression of matrix metalloproteases (J.F.S. and L.B., data not shown and reference 29). SW1353 cells were transfected with the 48 bp *Col2a1* reporter and then treated with vehicle, TNFα (10 ng/ml) or IL-1β (10 ng/ml) [Fig. 2(A)]. Enhancer driven luciferase expression was reduced approximately 60% by TNFα and 50% by IL-1β treatment. Similar numbers of cells were recovered following cytokine treatment as determined by DNA quantitation using Hoechst staining of duplicate cultures [Fig. 2(B)].

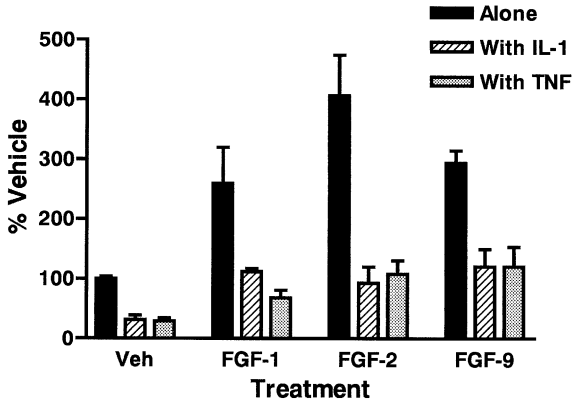

Fig. 3. FGFs block the down-regulation of *Col2a1* enhancer activity by cytokines. SW1353 cells were transfected, plated, and treated as in Fig. 2A, except for the additional cotreatment of the indicated FGF with IL-1β or TNFα. The relative light units have been normalized to percent vehicle control, with vehicle equal to 100%. Results correspond to the mean and standard error from triplicate wells of a representative study. Similar results were obtained in three independent experiments.

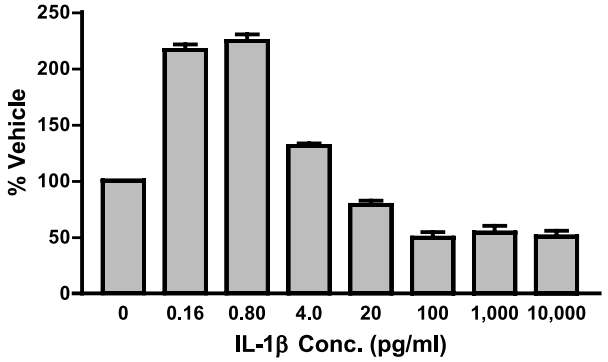

Fig. 4. The dynamic range of SW1353 biological response as a function of IL-1β concentration. SW1353 cells were transiently transfected with the 48 bp *Col2a1*Luc enhancer reporter and treated with increasing concentrations of IL-1β. After 24 h media were collected and luciferase activity measured as in Fig. 2A. Results correspond to the mean and standard error from triplicate wells of a representative study. Similar results were obtained in a duplicate study.

CYTOKINE-MEDIATED INHIBITION OF ENHANCER EXPRESSION CAN BE BLOCKED BY CO-TREATING CELLS WITH FGFS

As described earlier, SW1353 cells respond much like murine chondrocytes to FGF-1 and FGF-2, and we extend these findings to include FGF-9. Similarly, cytokines repress *Col2a1* enhancer activity in SW1353 cells. These results inspired us to address an important biological question—can FGF co-treatment prevent the functional consequences of IL-1β and TNFα on Sox9 activity and Col2 enhancer expression? The results of this might influence the consideration of FGF strategies for the treatment of joint damage resulting from inflammatory diseases. To address this, SW1353 cells transfected with the *Col2a1* reporter were cotreated with cytokine and with or without FGF-1, FGF-2, or FGF-9 (Fig. 3). The results of this experiment show that FGF-1, -2, and -9 can lead to a significant attenuation of IL-1β- and TNFα-mediated repression of *Col2a1* enhancer activity. This novel result indicates that pro-chondrogenic FGFs have the potential to antagonize cytokine driven down-regulation of cartilage matrix synthesis and suggests that Sox9 may play a role in integrating these responses.

EXPRESSION OF THE *COL2A1* ENHANCER REPORTER IS STIMULATED BY THE PRESENCE OF LOW LEVELS OF IL-1β

An increase in the expression of two Sox9 regulated genes, *Col2a1* and aggrecan, has been observed in both humans and dogs during the course of the osteoarthritic disease[30–32]. Expression of both Col2 and aggrecan is greatest in early to moderate disease and may reflect a response by the chondrocytes to attempt repair of the degenerating cartilage. Coincidentally, chondrocytes from OA patients with early to moderate disease also show expression of IL-1α and IL-1β and this expression declines with the progression of the disease[33]. Thus, IL-1 expression is coincident with Col2 expression. IL-1 found in osteoarthritic joint appears to be derived, at least in part, from chondrocytes[33] while macrophages produce large amounts of IL-1 found in the rheumatoid joint[34]. This suggests that IL-1 may not play the same role in the two diseases.

To investigate this apparent paradox, we analyzed the effect of varying IL-1β doses on the expression of the 48 bp *Col2a1* enhancer in transfected SW1353 cells. Surprisingly, very low levels of IL-1β (0.1–1 pg/ml) stimulated enhancer activity about ~200% (Fig. 4); although the magnitude of induction was modest, this response was consistently observed. *Col2a1* enhancer activity was decreased as the concentration of IL-1β was increased above 1 pg/ml, with maximal inhibition occurring between 20 and 100 pg/ml. Therefore, SW1353 cells appear to respond to a dynamic range of IL-1β levels with selective functional responses. In addition, we found no evidence of a significant additive or synergistic effect between low levels of IL-1β and FGFs in cotreatment experiments (data not shown).

COL2 ENHANCER ACTIVITY CORRELATES WITH RELATIVE SOX9 MRNA AND PROTEIN EXPRESSION IN SW1353 CELLS

Previous studies showed that *Col2a1* enhancer activity correlates with Sox9 expression. FGF-2 stimulates and cytokines repress the expression of Sox9 mRNA and protein in primary mouse chondrocytes[14,15], results that point to Sox9 as being a key intermediate for regulating expression of Col2 and aggrecan expression. Therefore, we analyzed the effect of chondrocyte growth factors as well as inflammatory cytokines on the expression of Sox9 mRNA in SW1353 cells using quantitative real-time PCR. Cells were cultured and treated with growth factors or cytokines as in Fig. 2A, and after 48 h cells were harvested and RNA prepared. Expression of Sox9 mRNA was stimulated approximately 1.5-fold by FGF-2 and 2-fold by FGF-1 or FGF-9, while TNFα and IL-1β decreased Sox9 expression to 60 and 47% of control, respectively (Fig. 5). Similar results were obtained by analyzing Sox9 mRNA after 24 h of treatment (data not shown). This suggests that Col2 enhancer activity is regulated, at least in part, by the level of Sox9 expression; a finding that is consistent with studies performed in mouse chondrocytes[14,15].

We next examined whether the correlation between Col2 enhancer activity and Sox9 mRNA levels could be extended to the expression of endogenous Sox9 protein in

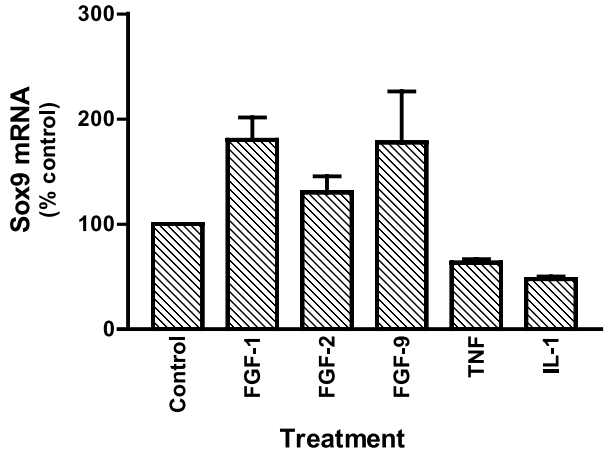

Fig. 5. The opposing effects of FGFs and cytokines on the expression of Sox9 mRNA in SW1353 cells. RNA was isolated from SW1353 cells treated for 48 h with vehicle (control), FGF-1 (200 ng/ml), FGF-2 (5.9 ng/ml), FGF-9 (200 ng/ml), TNFα (10 ng/ml), or IL-1β (10 ng/ml) as indicated. Sox9 expression was quantitated by Taqman and normalized to GAPDH expression. Data represents the mean and standard error from two or more experiments depending on the treatment group.

SW1353 cells. Cells were treated for 24 h with the indicated FGF, TNFα, IL-1β, or combination thereof, and cell extracts prepared for Western blot analysis. In order to normalize for any variability in the amount of total protein loaded, the Sox9 antibodies were stripped from the blot and the blot was probed with an antibody to GAPDH. Sox9 protein expression was normalized to GAPDH and then all treatments were compared relative to the vehicle. Expression of endogenous Sox9 is observed in the vehicle lane (Fig. 6, lane 1), although this requires more cell extract, greater sensitivity chemiluminescence reagents, and a longer exposure time when compared with that using overexpressed protein (see Fig. 1). Cells treated with FGF-1, FGF-2, and FGF-9 showed increased expression of Sox9 protein ranging from 150 to 260% of vehicle (Fig. 6 lanes 2, 3 and 4, respectively); while the levels of Sox9 protein were reduced by 80 and 90% following 24 h of treatment with IL-1β and TNFα, respectively (Fig. 6, lanes 5 and 6). At 48 h after cytokine treatment, Sox9 protein expression was again detectable but had not returned to untreated levels (data not shown). Thus, FGFs increase and cytokines reduce the mRNA, protein, and functional activity of endogenous Sox9.

As shown earlier (Fig. 3), we found that FGFs could antagonize the IL-1β and TNFα-mediated inhibition of the *Col2a1* enhancer activity in transfected SW1353 cells; a finding that could be explained by several different mechanisms including a reduction in Sox9 expression, Sox9 activity, and/or by Sox9-independent cross-talk between cytokine receptor and FGF receptor signaling pathways. To address these possibilities, we began by determining the effect of FGF-1, FGF-2, and FGF-9 on the IL-1β and TNFα-mediated down-regulation of Sox9 protein expression. As shown in Fig. 6, FGF-1, FGF-2, and FGF-9 cotreatment with IL-1β or TNFα, resulted in the preservation of Sox9 protein expression, albeit to different extents). This result is consistent with *Col2a1* enhancer activity in SW1353 cells in response to cotreatment with FGF and IL-1β or TNFα, where the basal enhancer activity was maintained by FGF cotreatment (see Fig. 3). Although FGF-1 and -2 are active, FGF-9 appears to have the greatest protective effect in both experiments, maintaining Sox9 levels and *Col2a1* reporter expression. Thus, FGF-9 may be particularly useful in counteracting joint damage mediated by inflammatory cytokines.

Discussion

In this study, we demonstrate that SW1353 cells model many Sox9 signaling pathways identified in primary murine chondrocytes. FGF-1 and FGF-2 were found to increase Sox9 mRNA levels and the corresponding activity of the Sox9-dependent 48 bp *Col2a1* enhancer reporter. This may have relevance to the activity of chondrocytes attempting the repair of cartilage damage or when considering strategies for therapeutic intervention. For example, Sox9 expression is elevated in the joints of mice attempting to repair damage induced by the expression of a mutant type IIA collagen gene[35]. In situ analysis showed that Sox9 expression corresponds with the most proliferative and metabolically active chondrocytes present in the repair tissue. Likewise, expression of endogenous FGF-2 is correlated with the repair of joint damage, as a neutralizing antibody to FGF-2 blocks the repair of full-thickness cartilage defects in a rabbit model, while the infusion of FGF-2 leads to improved repair[36]. Thus, Sox9 provides a direct link between the input of prochondrogenic growth factors and the expression of chondrocytic genes needed for repair.

We report the novel finding that FGF-9 stimulates Sox9 expression and activity in a human chondrocyte-like cell line. In agreement with our findings, FGF-9 and FGF-2 were also found to be the most potent FGF family members in stimulating growth and matrix production in chicken chondrocyte cultures[37]. As mentioned previously, FGF-9 expression is associated with the growth of benign cartilage nodules in the joints of chondromatosis patients. Taken together with our results, stimulation of the FGF-9/FGFR/Sox-9 signaling pathway may have therapeutic potential in stimulating the repair of cartilage damage. However, further studies are needed to define the full spectrum of FGF-9 biological functions. FGF-9 signals through both FGFR2c and FGFR3b[23], and mice lacking FGFR3 have severe achondroplasia[38]. However, no defects in cartilage or cartilage-derived tissues have been described for mice lacking FGF-9, but XY sex reversal and lung hypoplasia have been reported instead[39,40]. This suggests that additional FGFs can compensate for the loss of FGF-9 in developing cartilage, but such functional redundancy does not exist for FGFR3. The phenotypic similarity between the FGF-9 and Sox9 deficiency and male sex reversal is striking and raises the intriguing possibility that FGF-9/Sox9 signaling is required for the normal development of the male reproductive organs. The role of FGF-9 in reproductive development needs to be considered when assessing the therapeutic potential of FGF-9 for joint repair approaches.

The repression of Col2 expression by IL-1β is not unique to SW1353 cells and has been reported in immortalized human chondrocyte cell lines[18,41]. However, we present the original finding that SW1353 cells respond to increasing IL-1β levels with dynamic range of responses. The functional significance of this observation will depend on additional studies. However, it is worth considering whether stimulation of the 48 bp *Col2a1* enhancer by low levels of

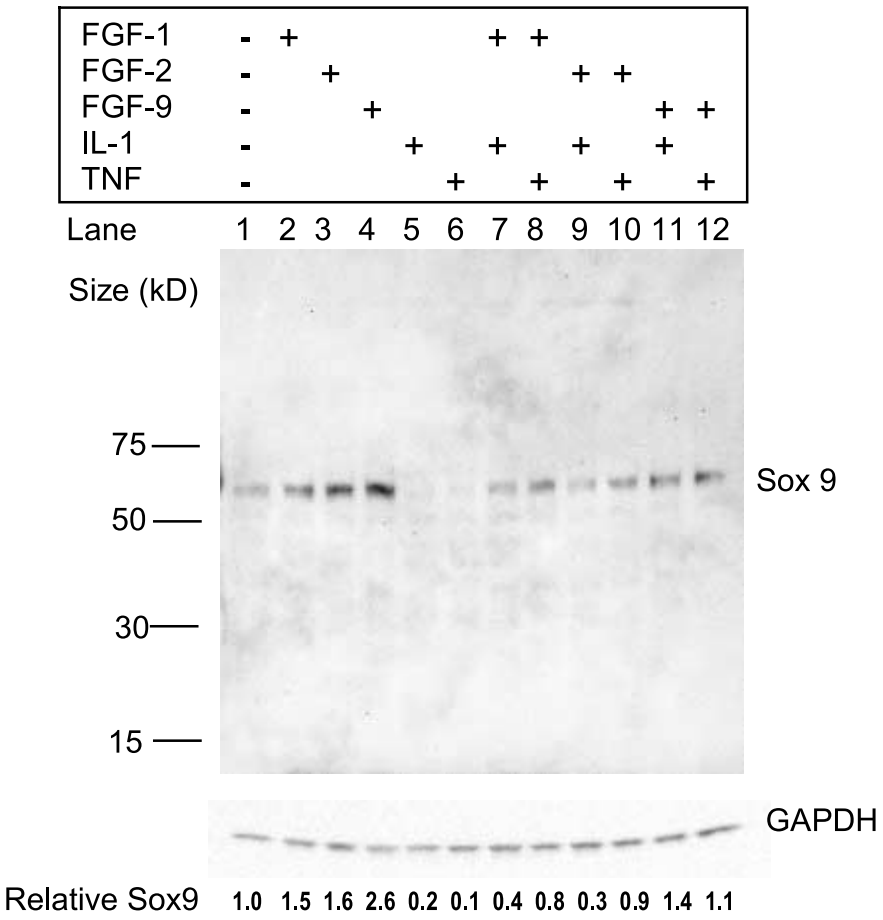

Fig. 6. Effect of FGFs, IL-1β, and TNFα on the expression of Sox9 protein analyzed by Western blot. Cell lysates were prepared from SW1353 cells treated for 24 h without or with FGFs and/or cytokines as in Fig. 2B. Whole cell lysates were prepared and equivalent amounts were used for Western blot analysis. The blot was probed with Sox9 affinity purified antisera. After imaging, the blot was stripped of the Sox9 antibody and probed with a GAPDH monoclonal antibody. Relative Sox9 protein expression was quantified as described in the Materials and methods section. Sox9 protein expression is increased by FGF-1, FGF-2, and FGF-9 (lanes 2–4), while IL-1β and TNFα diminish Sox9 protein expression (lanes 5 and 6). Addition of each FGF with TNFα or IL-1β results in the maintenance of Sox9 expression (lanes 7–12, as indicated).

IL-1 may relate to the paradoxical results from osteoarthritic joints, where chondrocyte IL-1 expression correlates with increased type II collagen synthesis[30,32]. In other words, do chondrocytes express low levels of IL-1 in an attempt to drive Col2 expression and stimulate repair?

In summary, we show that SW1353 cells conserve many of the Sox9 signaling pathways described in studies of murine chondrocytes. We extend these studies and present the novel finding that FGF-9 is a potent factor in regulating Sox9 expression and activity. We make the novel observation that FGF-1, -2, and -9 can attenuate or reverse Sox9 inhibition caused by pro-inflammatory cytokines. Furthermore, we demonstrate that SW133 cells react to increasing IL-1β levels with a dynamic range of responses. Therefore, we consider SW1353 cells to be a useful human cell model for studying Sox9 signaling pathways.

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